PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year)	
04 December 1997 (04.12.97)	
International application No.	**Applicant's or agent's file reference**
PCT GB97 01158	38630 JMD
International filing date (day/month/year)	**Priority date** (day/month/year)
24 April 1997 (24.04.97)	24 April 1996 (24.04.96)
Applicant	
QUIBELL, Martin et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

21 November 1997 (21.11.97)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
38630/JMD		
International application No	International filing date (day/month/year)	Priority date (day/month/year)
PCT/GB97/01158	24/04/1997	24/04/1996
International Patent Classification (IPC) or national classification and IPC		
C07K1/04		
Applicant		
PEPTIDE THERAPEUTICS LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☒	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Name and mailing address of the IPEA

Authorized officer

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB97/01158

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-105 as originally filed

Claims, No.:

1-15 as originally filed

Drawings, sheets:

1/17-17/17 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), [illegible] have not been examined in respect of:

☒ claims Nos. 4,5

☒ the said international application, or the said claims Nos. 4,5 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. ☐ As all the

☐ the parts relating to claims Nos.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2,3,6-15
	No:	Claims	1
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-3,6-15
Industrial applicability (IA)	Yes:	Claims	1-3,6-15
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

1. Reference is made to the following document:

   D1: Proc. 23rd Eur. Peptide Symp. **1995**:455-456

2. The present application is directed to "autodeconvoluting" libraries for the characterisation of an *active motif* in *any* group of compounds.

   D1, which considered representing the closest prior art, discloses "self-deciphering", orthogonal libraries which lead to the discovery of a potent V2 vasopressin antagonist. "Self-deciphering" is to be interpreted as being a synonym for "autodeconvoluting". Therefore, all technical features of present claim 1, as far as they are fulfilling the requirements of clarity (Article 6, cf point), are present in D1, i.e. the subject-matter of claim 1 is not new in the sense of Article 33(2) PCT.

   Furthermore, D1 discloses the suitability of the described type of self-deciphering libraries for *any* group of compounds, i.e. methods using this type of libraries are not restricted to peptides or oligonucleotides.

   The problem underlying the present application can be regarded as to provide further particular "autodeconvoluting" (*sive* self-deciphering or self-decoding) libraries to identify an inhibitor of an enzyme or a binding motif of an antigen etc.

   Since the general principle of self-decoding libraries is known from D1 (for three variables) it appears not to involve an inventive step to adapt the principle to four variable entities. The skilled person is enabled to develop any library format as presently claimed to screen for a desired activity from the teachings of D1. Therefore, claims 2-15 are not in accordance with the requirements of Article 33(3) PCT with respect to inventive step.

   [illegible] invention" according to Rule 6.3(a) PCT.

Independent claim 1 is not in the two-part form -and therefore not in accordance with Rule 6.3(b) PCT-, which in the present case would be appropriate, with those features known in combination from the prior art (document D1) being placed in a preamble (Rule 6.3(b)(i) PCT) and with the remaining features being included in a characterising part (Rule 6.3(b)(ii) PCT).

3. The problem concerning the screening and rapid identification of an desired effector out of a large number of compounds has been solved already by the disclosure of the principle of "self-decoding" libraries (D1).

   The target classes of compounds of claims 8-12 which the method of claim 3 is directed to are not anymore linked in such a way by a special technical feature in the sense of Rule 13(2) PCT that they represent a single inventive concept (non-unity *a posteriori*; Rule 13(1) PCT). Every single library claimed (and methods using this library) concerning a particular target class of compounds is considered as a separate invention.

4. The terms "*active moiety*" and "*active motif*" used in claim 1 are vague and unclear and leave the reader in doubt as to the meaning of the technical features to which they refer, thereby rendering the definition of the subject-matter of said claim unclear (Article 6 PCT).

5. The subject-matter of present claims 4 and 5 is directed to performing a pure mental act, i.e. a deciphering procedure. This subject-matter is excluded from international preliminary examination (Article 34(4)(b); Rule 67.1(iii) PCT).

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
REDDIE & GROSE
Attn. DAVIES, JONATHAN M.
16, Theobalds Road
LONDON WC1X 8PL
UNITED KINGDOM

Date of mailing (day/month/year) 13. 10. 97

Applicant's or agent's file reference: 38630/JMD	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No.: PCT/GB 97/01158	International filing date (day/month/year): 24/04/1997
Applicant: PEPTIDE THERAPEUTICS LIMITED et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in many Offices, even later).

Name and mailing address of the International Searching Authority:	Authorized officer:
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk	Alfredo Prein

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter

[illegible] to the applicant. However, if the language of the international application is English, the letter must be in English. If the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3 [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4 [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/01158

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K1/04 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. MELDAL ET AL.: "Portion-mixing peptide libraries of quenched fluorogenic substrates for complete subsite mapping of endoprotease specificity" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 8, 12 April 1994, WASHINGTON US, pages 3314-3318, XP002042095 see page 3317, right-hand column, paragraph 2 - page 3318, right-hand column, paragraph 1 ---	1,2
A	WO 95 34575 A (SYNTHETIC PEPTIDES INC) 21 December 1995 see the whole document ---	1,3,9-12
	-/--	

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu-

Date of the actual completion of the international search

30 September 1997

Date of mailing of the international search report

13. 10. 97

Name and mailing address of the ISA
European Patent Office
Fax (+31-70) 340-3016

Authorized officer

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/01158

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534575 A	21-12-95	AU 2805995 A	05-01-96
WO 9405394 A	17-03-94	US 5585275 A	17-12-96
		US 5591646 A	07-01-97
		AU 4844593 A	29-03-94
		AU 6393994 A	14-09-94
		JP 8507602 T	13-08-96
		WO 9419694 A	01-09-94

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
38630/JMD		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 97/01158	24/04/1997	24/04/1996
Applicant		
PEPTIDE THERAPEUTICS LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

   [ ] filed with the international application.

   [X] furnished by the applicant separately from the international application,

      [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

   [ ] Transcribed by this Authority

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant

   [ ] the text has been established by this Authority to read as follows

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international

[illegible]

[illegible] as suggested by the applicant [X] [illegible]

[ ] because the applicant failed to suggest a figure

[illegible]

INTERNATIONAL SEARCH REPORT

[redacted]national Application No
[redacted]CT/GB 97/01158

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K1/04 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. MELDAL ET AL.: "Portion-mixing peptide libraries of quenched fluorogenic substrates for complete subsite mapping of endoprotease specificity" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 8, 12 April 1994, WASHINGTON US, pages 3314-3318, XP002042095 see page 3317, right-hand column, paragraph 2 - page 3318, right-hand column, paragraph 1 ---	1,2
A	WO 95 34575 A (SYNTHETIC PEPTIDES INC) 21 December 1995 see the whole document ---	1,3,9-12
	-/--	

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

° Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled

30 September 1997

Name and mailing address of the ISA

Authorized officer

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/01158

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	WO 94 05394 A (ARRIS PHARMACEUTICAL CORP) 17 March 1994 see claims; examples ---	1,3,9-12
A	B. Déprez et al.:" Self-decifering, orthogonal combinatorial libraries of soluble organic compounds: Discovery of a potent V2 vasopressin antagonist" in: XP002042096 Peptides 1994 Proceedings of the Twenty-Third European Symposium September 4-10, 1994, Braga, Portugal ed. HLS Maia; pub. ESCOM, Leiden, NL, 1995, pages 455-456 ---	1,3,11
P,X	A.F. SPATOLA AND Y. CROZET: "Rediscovering an Endothelin Antagonist (BQ-123): A Self-Deconvoluting Cyclic Pentapeptide Library" JOURNAL OF MEDICINAL CHEMISTRY, vol. 39, no. 19, 13 September 1996, WASHINGTON US, pages 3842-3846, XP002042200 see page 3844, left-hand column, paragraph 4; table 1 -----	1,3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/01158

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534575 A	21-12-95	AU 2805995 A	05-01-96
WO 9405394 A	17-03-94	US 5585275 A	17-12-96
		US 5591646 A	07-01-97
		AU 4844593 A	29-03-94
		AU 6393994 A	14-09-94
		JP 8507602 T	13-08-96
		WO 9419694 A	01-09-94

Auto-Deconvoluting Combinatorial Libraries

The present invention relates to the field of apparatus and methods which provide the rapid generation of structure/activity relationships using auto-deconvoluting combinatorial libraries, which facilitate the invention of novel active compounds. The invention provides apparatus and methods which can be used for the rapid generation of structure/activity relationship (SAR) data, and, therefore, the characterisation of the active motif of any group of compounds.

The invention provides libraries of compounds which interact with an active moiety, and apparatus and methods to identify such compounds. The active moieties may be (but are not limited to) enzymes, receptors, antibodies, etc. The interaction of the active moiety with the compounds of the library may be (but is not limited to) the interaction of a substrate or inhibitor with an enzyme, the interaction of a ligand with a receptor, the interaction of an antigen or antigenic epitope with an antibody, etc.

The following list illustrates a number of enzyme classes and subclasses:

Enzyme Classes

1. Oxidoreductase

d) catalase

2. Hydrolase

f) peptidase (proteolytic enzyme)

3. Transferase

g) aminotransferase

h) kinase

i) glucosyltransferase

4. Lyase

j) decarboxylase

k) dehydratase

5. Isomerase

l) racemase

m) mutase

6. Ligase

n) synthetase

o) carboxylase

Proteases of interest include (but are not limited to):

1. Aspartyl proteases, such as renin, HIV, cathepsin D and cathespin E etc.

2. Metalloproteases, such as ECE, gelatinase A and B,

4. Serine proteases, such as thrombin, factor VIIa, factor Xa, elastase, trypsin.

5. Threonyl proteases, such as proteasome S.

Proteases

Many therapeutically useful drugs act as enzyme inhibitors. In particular, proteolytic enzyme inhibitors have been the focus of much attention in the pharmaceutical industry, because they play a variety of roles in a multitude of biological systems. Their proteolytic activities are related to processes ranging from cell invasion associated with metastatic cancer to evasion of an immune response, as seen in certain parasitic organisms; from nutrition to intracellular signalling to the site-specific proteolysis of viral proteases and eukaryotic hormone-processing enzymes. However, the traditional random screening methods for the identification of lead molecules as inhibitors of proteolytic enzymes are often laborious and time-consuming. Therefore new and efficient methods which can accelerate the drug discovery process are greatly in demand.

We consider that proteases contain an active catalytic site which tends to become increasingly activated as the recognition pockets (S_1 and S_1 etc' and (S_1' and S_1' etc'

inhibitors. Therefore, we have devised a combinatorial method for the rapid identification of these binding motifs which will greatly expedite the synthesis of inhibitors of a variety of proteolytic enzymes such as aspartyl proteases, serine proteases, metallo proteases and cysteinyl proteases.

The use of a fluorescence resonance energy transfer (FRET) substrate for the analysis of proteolytic enzyme specificity was first published by Carmel.[1] Since then many different quenched fluorogenic substrates for measuring enzyme inhibition have been described in the literature.[2-11] These substrates contain a fluorophore, F, in a P position (*vide supra*), which is quenched by another group, Q, present in a P' position (*vide supra*) and separated from F by the scissile bond. The advantage of the positioning of these residues, F and Q, is that cleavage of a peptide bond occurs between the two natural residues and, therefore, represents a more natural hydrolytic event rather than the cleavage and release of a C-terminal chromophore.

For example, Bratovanova and Petkov[12] have synthesised fluorogenic substrates from peptide 4-nitroanilides. N-acylation of peptide 4-nitroanilides with the aminobenzoyl (ABz) group yielded substrates that are internally quenched by the presence of the 4-nitroanilide moiety. Upon hydrolysis of the aminoacyl-4-nitroanilide bond, the [illegible] ABz group is released attached either

Immobilised libraries, where substrates are attached to a polymer or biopolymer support, have also been used for mapping protease binding sites.[9] Singh et al. reported recently that enzymatic substrate activity of 38 selected octapeptides attached *via* a linker to controlled pore glass is predictive of the same activity of similar peptides in solution. However, these results are preliminary and only for a specific example. Therefore, it is not clear whether immobilised substrates attached to polymers can reliably replace soluble substrates in mapping the hindered protease binding sites, especially since the hydrophilic or lipophilic nature of the polymer and the size of the interstices within the polymer are bound to influence the reaction between the enzyme and its substrates.

Mixtures of internally quenched, fluorogenic substrates have also recently been described in which the quencher group, Q, is 2,4-dinitrophenyl (Dnp) and is attached to the P side of the scissile bond, while the fluorogenic group, is N-methyl anthranilic acid (Nma) and is attached to the P' side.[14]

Examples of other Donor-Acceptor Chromophore Pairs that have been applied to Biological Systems are shown in Table 1.

Table 1 : Donor-Acceptor Chromophore Pairs That Have Been Applied To

IANBD	IDPX		IANBD
IAEDANS	DDPM	NBD	SRH

DNSM	LY	ISA	TNP
IAEDANS	IANBD	Dansyl	ODR
E-A	F_2DNB	DANZ	IAF
Pyrene	Bimane	FNAI	EITC
ANAI	IPM	NBD	LRH
IAANS	IAF	IAF	EIA
ϵ-A	F_2DPS	FITC	ENAI
ϵ-A	DDPM	Proflavin	ETSC
IAEDANS	TNP	CPM	TNP-ATP
MNA	DACM	IAEDANS	IAF
PM	NBD	CPM	Fluorescein
FITC	TNP-ATP	IAEDANS	FITC
DANZ	DABM	FITC	TMR
NCP	CPM	IAF	TMR
NAA	DNP	CF	TR
LY	TNP-ATP	CPM	FTS
IAF	diI-C_{18}	ϵ-A	TNP-ATP
IAF	TMR	CPM	EM
FMA	FMA	LY	EM
PM	DMAMS	FITC	EITC
mBBR	FITC	IAEDANS	DiO-C_{14}
mBBR	DABM	IAF	ErITC
ϵ-A	NBD	FITC	EM
Pyrene	Coumarin	FITC	ETSC
IPM	FNAI	FITC	ErITC
IAEDANS	DABM	BPE	CY5

ANAI, 2-anthracene N-acetylimidazole; BPE, B-phycoerythrin; CF, carboxyfluorescein succinimidyl ester; CPM, 7-doethylamino-3-(4'maleimidylphenyl)-4-methylcoumarin; CY5, carboxymethylindocyanine-N-hydroxysuccinimidyl ester; diI-C_{18}, 1,1'-dioctadecyl-3,3,3'3'-tetramethyl-indocarbocyanine; DiO-C_{14}, 3,3'-ditetradecyloxacarbocyanine; DABM, 4-dimethylaniniphenylazo-phenyl-4'-maleimide; DACM, (7-(dimethylamino)coumarin-4-yl)-acetyl; DANZ, dansylaziridine; DDPM, N-(4-dimethylamino-3-5-dinitrophenyl)maleimide; DACM, di-methylamino-4-maleimidostilbene; DMSM, N-(2,5-dimethoxystiben-4-yl)-maleimide; DNP, 2,4-dinitrophenyl; ϵ-A, 1,N⁶-ethenoadenosine;EIA, 5-(iodoacetamido)eosin, EITC, eosin-5-isothiocyanate; ENAI, eosin N-acetylimidazole; EM, eosin maleimide; ErITC, erythrosin-5'-isothiocyanate; ETSC, eosin thiosemicarbazide; F_2DPB, 1,5-difluoro-2,4'dinitrobenzene; F_2DPS, 4,4'-difluro-3,3'dinitropheylsulphone; FITC, fluorescein N-acetylimidazole; FTS, fluorescein thiosemicarbazide; IAANS; 2-(4'-iodoacetamidoanilino)naphthalene-6-sulphonic acid; IAEDANS, 5-(2-

[illegible]

(iodoacetamido)salicylic acid;LRH, lissaminerho-2,1,3-benzoxadiazol-4-yl; NCP, N-cyclohexyl-N'-(1-pyrenyl)carbodiimide; ODR, octadecylrhodamine; PM, N-(1-pyrene)-maleimide; SRHsulphurhodamine; TMR, tetramethylrhodamine; TNP, trinitrophenyl; TR, Texas red.

from Wu, P. and Brand, L. 1994. Anal. Biochem. 218, 1-13.

The specificity of soluble peptide libraries have been determined.[16,17] Berman et al. described[18] an HPLC mass spectrometry technique in which 6 mixtures of 128 peptides were synthesised which were N-terminally labelled with the Dnp group in order to allow UV monitoring on the HPLC. The disadvantage of this approach is that each assay mixture has to be individually analysed, because no fluorogenic substrate is revealed, and that the effective concentration of each separate component is limited by the size of the mixture because of overall solubility factors.

Drevin et al.[19] have suggested the use of individually synthesised fluorogenic substrates for the determination of enzyme activity using a chromophore which chelates lanthanide ions. Garmann and Phillips have suggested the use of FRET substrates in which the fluorogenic and quencher moieties are attached via thiol or amino functional groups after the peptide has been synthesised, but this has the disadvantage that they are not in library form and that these functional amino and thiol groups need to be selectively revealed after the peptide has been synthesised. Wang et al. have suggested the use of the

The above methods which have used FRET techniques for the mapping of the active site around a specific protease suffer from one or more of the following disadvantages:

i. because of general aqueous insolubility they do not produce mixtures of compounds in a form suitable for high throughput screening in aqueous solution.

ii. the derivatised compounds cannot be prepared in combinatorial library form using solid phase techniques.

iii. the mixtures which have been used[**] were not self-decoding, and needed time-consuming deconvolutive resynthesis for identification of the active molecules.

Kinases

Protein kinases are intracellular enzymes that play key roles in cell growth, differentiation and inter-cell communication. Aberrant protein kinase activity has been implicated in many disease states including several forms of cancer and severe-combined immunodeficiency disease. All serine/threonine and tyrosine protein kinases have a region of approximately 300 amino acids known as the catalytic subunit which has evolved from a common ancestor kinase (Hunter et al, 1991). Crystal structure determination of several kinases has shown that they all have a common bi-lobal structure. The amino-terminal part of the subunit encodes a small lobe responsible for the binding of ATP, whereas the carboxy-terminal residues

brought together allowing transfer of the ATP γ-phosphate to the amino acid acceptor on the protein substrate. The protein/peptide binding groove stretches across the face of the large lobe between two α-helices and under the small lobe. This groove therefore contains the residues important for substrate specificity of the kinase. Protein kinases are arranged in kinase cascades within the cell, providing the ability for signal amplification in post-transduction pathways. This amplification relies on the upstream kinase specifically activating its downstream partner. For this reason, protein kinases have developed remarkable substrate specificities which prevent unwanted crosstalk between different kinase cascades. We believe that such substrate specificity can be exploited in the design of selective protein kinase inhibitors.

Existing protein kinase inhibitors

Non-peptide inhibitors

Most available non-peptide protein kinase inhibitors do not target the substrate binding region, but compete with ATP for enzyme binding. These inhibitors can be engineered using conventional medicinal chemistry to demonstrate specific enzyme selectivity. An example of this strategy was the modification of the fungal metabolite staurosporine, a general protein kinase inhibitor, to the bis-indolyl-maleimides Ro 31-7549 and Ro 31-8425 (fig. 17) which are selective protein kinase C inhibitors (Muid et al, 1991). Unfortunately, the potency of ATP antagonist inhibitors can be dramatically reduced in certain intracellular compartments due to the high

The aromatic compound erbstatin (fig. 18) produced by certain Streptomyces strains was identified in screens for reversal of protein tyrosine kinase-induced cell transformation. Erbstatin was subsequently shown to inhibit the EGFR kinase *in vitro* with a k_i of 5.58 μM and typical lineweaver-Burke competitive kinetics against a peptide substrate, while remaining non-competitive with ATP (Umezawa and Imoto, 1991). The compound is readily inactivated in calf serum and so could not be considered as a serious candidate for therapeutic use. Attempts have been made to both increase the stability of erbstatin, by replacement of the N-formyl moiety, and bias the inhibition towards specific tyrosine kinases using a systematic modification of all parts of the molecule. These synthetic erbstatin derivatives are known as tyrphostins (tyrosine phosphorylation inhibitors) and are mainly used as tools in enzyme/signal transduction research, but have had limited success in animal models of disease (see Levitzki and Gazit, 1995 for an extensive review).

Pseudosubstrate inhibitors

Research into the structure and function of protein kinases uncovered a natural method of regulating catalytic activity known as pseudosubstrate inhibition. This method uses sequences, either on the same molecule as the catalytic site (protein kinase C) or on a regulatory subunit molecule (cyclic AMP-dependent protein kinase)

a phospho-acceptor results in the formation of a good substrate sequence. It is for this reason that the sequences are known as pseudosubstrates. Peptides based on pseudosubstrate motifs have been used as tools for inhibition of specific proteins kinases such as cyclic cAMP-dependent protein kinase (PKA), protein kinase C (PKC), myosin light chain kinase (MLCK) and calmodulin-dependent protein kinase II (Kemp *et al*, 1994). Pseudosubstrate peptides of less than 20 amino acids have been shown to be potent low nanomolar inhibitors *in vitro* with pseudosubstrate-based peptides of six amino acids inhibiting protein kinases at less than one micromolar (Kemp *et al*, 1991).

It is clear that there is scope for a small molecular weight inhibitor of protein kinases that competes with substrate for binding to the active site. A rational and peptide proven approach would be to base these inhibitors on substrate molecules which can specifically access the catalytic site. By focusing on pseudosubstrates, this approach would also have the advantage of "in-built" specificity, an element which is generally missing when leads are identified from random screening approaches.

Protein kinase inhibitors in disease treatment

Currently, there are no specific protein kinase inhibitors available for clinical use. Nevertheless, the potential for such compounds is clear with every major

Prototypic kinase inhibitor compounds are widely available from many companies, and are used as tools for *in vitro* signal transduction research. Several have formed the basis for extensive medicinal chemistry programmes designed to engineer both selectivity and enhanced activity into these compounds. A number of examples have been reported which indicate that this strategy has had some success in producing compounds active both *in vitro* within cell assay systems, and in animal models of disease. Staurosporine analogues (CGP41251 and UCN-01) have been used to show anti-tumour activity in vivo (Meyer *et al*, 1989; Akinagaka *et al*, 1991) and selective PKC inhibitors have been shown to work in animal models of acute inflammation (Bradshaw *et al*, 1993). Moreover, the BMN tyrphostin AG490 has been shown to inhibit lymphoblastic leukaemia growth in animal models, correlating with its ability to inhibit the non-receptor intracellular tyrosine kinase JAK-2 (Meydan *et al*, 1996). These promising preclinical studies have yet to be translated into the clinic, and it is possible that unforeseen toxicological problems, perhaps related to compound specificity, could be delaying their progress.

During the past few years, there has been an explosion of knowledge relating to kinase structure, function and activity. New kinases have also been identified, some of which have a sufficiently restricted cellular distribution that one could envisage that a specific inhibitor would

promise of new approaches for the development of kinase inhibitors to treat a range of conditions.

Method of Protein Kinase Inhibitor Design

Generation of initial substrate information

A technique has recently been developed by L. Cantley's laboratory to provide, consensus peptide kinase substrate information (Songyang et al, 1994). Firstly, a degenerate peptide library of peptides with a central phospho-acceptor such as tyrosine or serine/threonine flanked by four unknown amino acids on each side is synthesised. The library is then phosphorylated by the protein kinase of interest and phosphorylated peptides isolated by DEAE-sephacel and ferric chelation chromatography. The phosphopeptide mixture is then sequenced and the frequency of each amino acid at every position -4 to +4 assessed to give a preferred substrate sequence. These studies have yielded consensus substrate information, and do not allow a detailed analysis of particular preferences for neighbouring residue interactions. Similarly, the analysis was performed solely with natural amino acids. Filamentous phage are now also being used to generate substrate information (Miami, Biotechnology Conference, 1996). Substrate information can also be obtained from knowledge of the physiological substrates for the kinase in vivo.

Figures 1 to 14 inclusive exemplify component distributions in the plates of a library matrix;

Figure 15 illustrates a reaction scheme for production of compound number 4: Boc-Val-Ala-Leu-H wherein

i. isobutylchloroformate, N-methylmorpholine, then N,O-dimethylhydroxylamine HCl, THF.

ii. HCl, dioxan. iii. isobutylchloroformate, N-methylmorphline, then Boc-Ala-OH, THF.

iv. HCl, dioxan. v. Boc-Val-Osuc, N-methylmorpholine, DMF. vi. LAH;

Figure 16 illustrates a reaction scheme for production of active inhibitors of *Der PI*.

Figure 17 shows the molecular structure of the Roche protein kinase C inhibitors. A; Ro 31-8425/002, 3-[8(RS)-(aminomethyl)-6,7,8,9-tetrahydropyridol[1,2-a]indol-10-yl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione hydrochloride. B; Ro 31-7549/001, 3-[1-(3-aminopropyl)-3-indolyl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione hydrochloride.

Figure 18 shows the molecular structure of the protein tyrosine kinase inhibitor erbstatin, isolated from

Brief Description of the Invention

The present invention relates to the field of apparatus and methods which provide the rapid generation of structure-activity relationships using auto-deconvoluting combinatorial libraries, which facilitate the invention of novel active compounds.

We describe herein apparatus and methods which can be used for the rapid generation of structure-activity relationship (SAR) data and, therefore, the characterisation of the active motif of any group of compounds.

The invention provides two orthogonal sets of mixtures of compounds in solution providing two complementary combinatorial libraries indexed in two dimensions for autodeconvolution. These are referred to as primary and secondary libraries.

The general concept of two orthogonal sets of mixtures indexed in two dimensions can be applied to various permutations of numbers of wells, plate layout, number of permutations per mixture etc. However, according to the invention the numerical interrelationship is defined as indicated below for libraries containing compounds with four variable groups B,C,D and E.

General Deconvolution Formulae

1) Primary and Secondary plates preferably have the same number of compounds per well [X]: otherwise there are two values, having X_1 and X_2 respectively.

2) The primary library comprises [np] plates.

If Rp.Cp=Rs.Cs, then the number of plates in the secondary library is also [np]. If not, the number of plates in the secondary library [ns] is:

$$ns = \frac{Rp.Cp}{Rs.Cs} . np$$

eg. A primary library of np=4, Rp=8, Cp=10 can be set out in an Rs=4, Cs=5 secondary library with the number of plates equal to:

$$ns = \frac{8 \times 10}{4 \times 5} . np$$

= 16 plates.

Number of compounds per well

-Bb-Cc-Dd-np(Ee)-

$k = b.c.d.np.e$ (2)

When number of wells on a plate = [N], number of compounds per well = [X] and number of plates = [np]:

$k = X.N.np$ (3)

However, number of wells [N] is also defined by the number of rows [Rp] and number of columns [Cp]:

$N = Rp.\ Cp$ (4)

Combining (3) and (4):

$k = X.Rp.Cp.np$ (5)

Combining (2) and (5):

$b.c.d.np.e = X.Rp.Cp.np$ (6)

Cancelling [np] from both sides of the equation:

$b.c.d.e = X.Rp.Cp$ (7)

left side must be equal in number to the number of rows [Rp]. So:

$$[Cp]^{-1}.\ Rp.e = X.Rp.Cp \qquad (8)$$

Cancelling [Cp] and [Rp] from both sides of the equation:

$$Cp.e = X \qquad (9)$$

where [e] is the number of variants along a fixed row; and

if Rp=Cp, then Rp.e = X.

Example

for a 10 x 10 x 8 x 8 format over 4 plates:

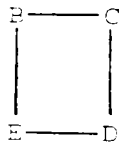
$$np.e = 8 \Rightarrow e = 2$$

$10 \times 2 = X$

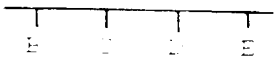
$X = 20.$

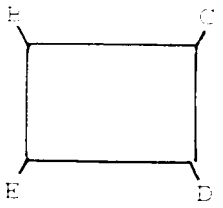
From an understanding of the general deconvolution formulae shown above, those skilled in the art will readily appreciate that the advantageous results of self-deconvolution according to the invention are obtainable utilising a number of different arrangements of wells, plate layouts, mixtures etc and that such variants on the preferred embodiment illustrated herein are intended to be within the scope of the present invention.

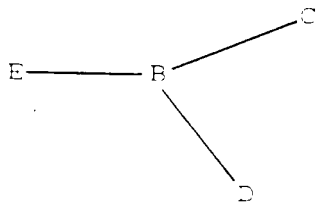
The molecules may be cyclic or alicyclic. They may be linear or cyclic on the same structure

B-C-D-E or 

or BCDE can be on a central scaffold (linear or cyclic)



or 



Where the symbol -B-C-D-E- or -B-C-D-nE- is used herein, it is to be understood to include all of these linear and cyclic variants within its definition.

At least one but not all the bonds between B, C, D, E need to be scissile. Non scissile bonds may include sulphonamide, urea, aminomethylene.

Several non-limiting examples of "scaffold" molecules are shown in Table 2, in which substituents R_1-R_4 correspond to possible variable groups B, C, D and E.

In a first aspect the invention provides novel compounds represented by the formula A-B-C-D-nE-F [I] in which;

A represents a fluorescer internally quenched by F;

B, C, D, and E represent groups such that the scissile bond between any two of these groups is a suitable bond;

F represents a quencher capable of internally quenching the fluorescer A; and

n represents an integer between 1 and 4 inclusive.

In some embodiments the suitable bond is an unsubstituted amide bond (see Example 1); in other embodiments the suitable bond is an ester bond (see Example 2).

In preferred embodiments B, C, D, E are amino acids or hydroxy acids. That is a molecule with an amine or hydroxy terminus and a carboxylic acid terminus. The amine/hydroxy group may be positioned on the same carbon atom or separated by a number of atoms and atom types.

In a second aspect the invention provides a combinatorial library of FRET compounds comprising a mixture of compounds of formula [I].

In a third aspect the invention provides for the use of such a combinatorial FRET library in a method which provides rapid generation of structure-activity relationships (SAR) which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme. According to this method an identified substrate can be synthesised and used in biological assay for proteolytic enzymes. Novel substrates are included in the scope of the invention.

In a forth aspect the invention provides for the use of

In a fifth aspect the invention provides a method which comprises the identification of an enzyme inhibitor or inhibitors wherein a FRET compound which has been

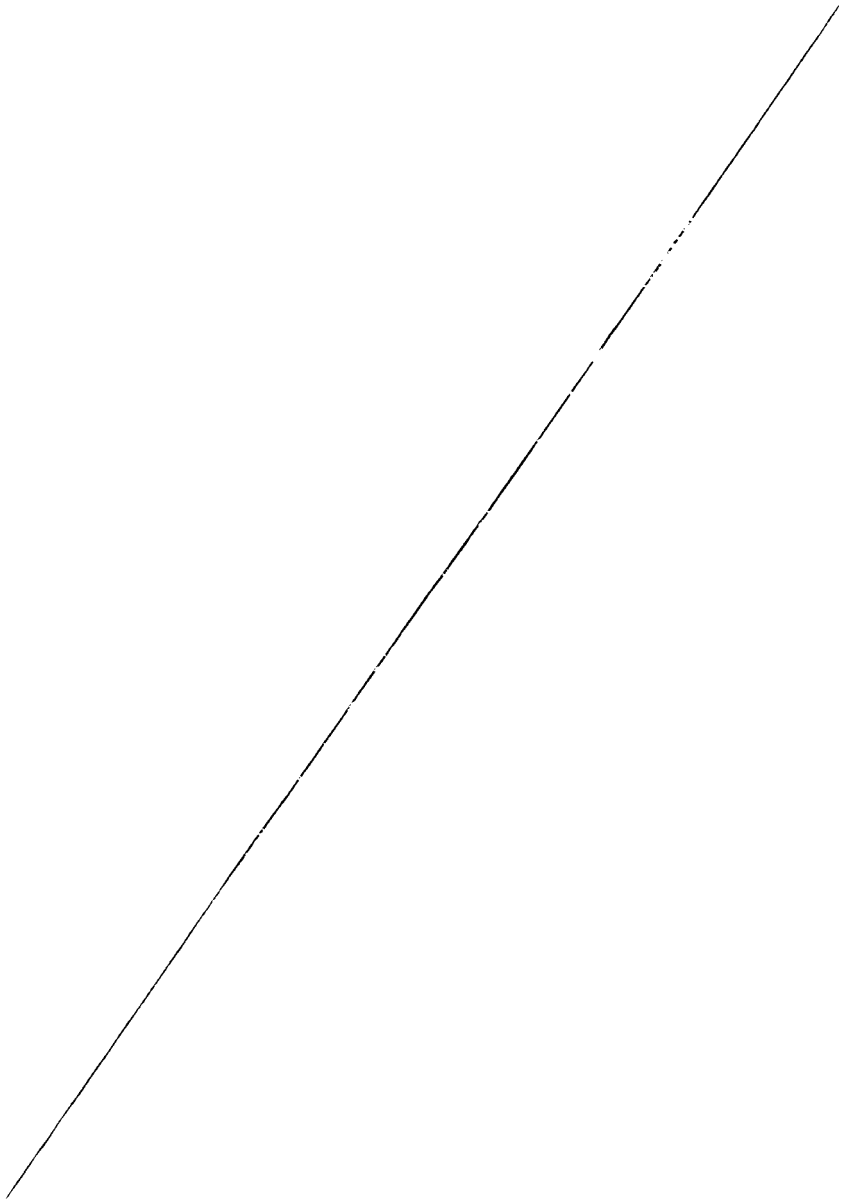
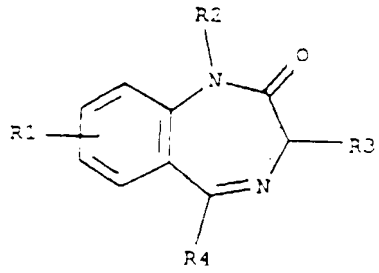
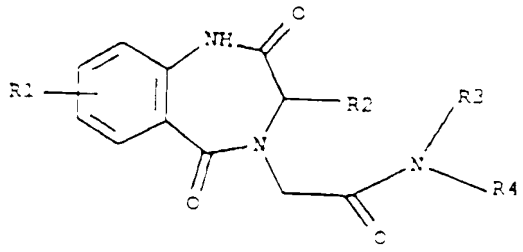
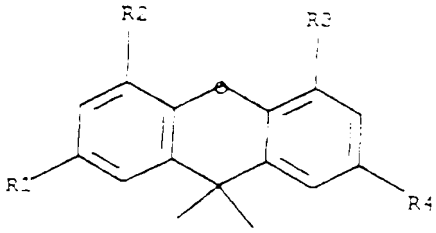
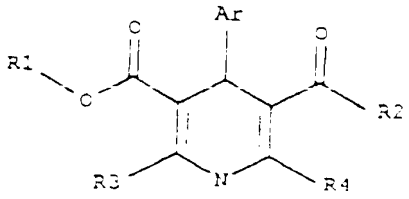


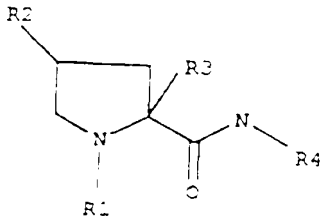
TABLE 2

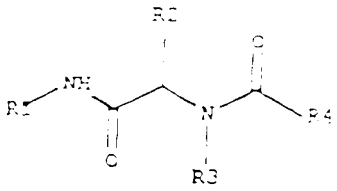


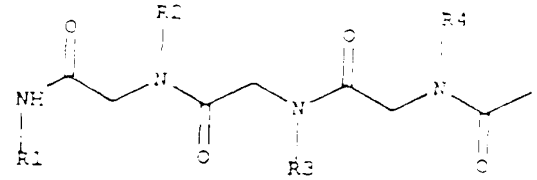


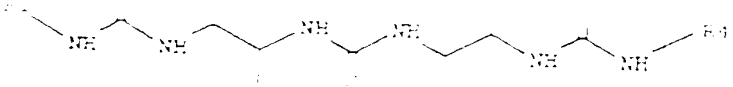












identified as a substrate is used in an inhibition assay with the enzyme separately against a panel of possible inhibitors.

In a sixth aspect the invention provides a set of compounds which comprises two complementary FRET compound libraries. Such a set will be referred to hereafter as "apparatus" because it allows for the screening or assay method for identifying substrates or inhibitors of proteolytic enzymes. This set of compounds constituting an apparatus is capable of providing an auto-deconvoluting combinatorial library as will be described below.

In a seventh aspect the invention provides a method of identifying and synthesising an inhibitor of a proteolytic enzyme which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules, deconvoluting the library to find a substrate or substrates for the enzyme and synthesis of an inhibitor based on the substrate or substrates. The direct product of this method is one or more novel proteolytic enzyme inhibitors.

In an eighth aspect the invention provides an inhibition assay which uses a FRET molecule, which has been identified as a substrate for the enzyme, wherein the

In a ninth aspect the invention provides a complementary pair of compound libraries L1 and L2 which constitute a set containing compounds of formula:

Aa-Bb-Cc-Dd-n(Ee)-Ff-Gg

giving a x b x c x d x e x f x g = Mn compounds in each library, there being a predetermined number (P1, P2) of mixtures each consisting of a predetermined number (Q1, Q2) of individual identifiable compounds in each library, wherein both L1 and L2 contain the same Mn compounds, but wherein any two compounds which are found together in one mixture of Q1 compounds of L1 are not found together in any one of the P2 mixtures of L2.

In a tenth aspect the invention provides a method of screening for enzymic activity using the libraries L1, L2 described above in which the P1 mixtures of L1 and the P2 mixtures of L2 are each placed separately into individual wells of well plates, the well plates having wells arranged in a format adapted to allow deduction of a unique active compound formula from the presence of activity in one well of L1 and one well of L2.

The apparatus of the invention preferably comprises two complementary compound libraries, L1 and L2, each containing n x 1000 compounds of the invention, of the

A = a fluorescor internally quenched by F, preferably an unsubstituted or substituted anthranilic acid derivative, connected by an amide bond to B

B, C, D, E, are natural or unnatural amino acid residues connected together by suitable bonds, although B, C, D and E can be any set of groups, provided that the scissile bond between D-E is an unsubstituted bond.

F = a quencher capable of internally quenching the fluorescor A, preferably an unsubstituted or substituted 3-nitrotyrosine derivative.

G = optionally present and is a hydrophilic moiety, preferably an aspartyl amide moiety. If present, G advantageously ensures that all compounds in the library are imparted with aqueous solubility. Also, G should not be a substrate for any type of enzyme.

n = any integer between 1 and 4 inclusive.

In an alternative, the scissile bond could be between B-C or C-D.

(Note that A and F herein correspond generally and respectively to moieties F and Q of the prior art referred to above).

The numbers represented in subscript following residues B, C, D and E refer to the number of possibilities from which

$A\text{-}B_1\text{-}C\text{-}D\text{-}E_1\text{-}F\text{-}G$

$A\text{-}B_2\text{-}C\text{-}D\text{-}E_1\text{-}F\text{-}G$

$A\text{-}B_3\text{-}C\text{-}D\text{-}E_1\text{-}F\text{-}G$

$A\text{-}B_4\text{-}C\text{-}D\text{-}E_1\text{-}F\text{-}G$

$A\text{-}B_5\text{-}C\text{-}D\text{-}E_1\text{-}F\text{-}G$

$A\text{-}B_6\text{-}C\text{-}D\text{-}E_1\text{-}F\text{-}G$

$A\text{-}B_7\text{-}C\text{-}D\text{-}E_1\text{-}F\text{-}G$

$A\text{-}B_8\text{-}C\text{-}D\text{-}E_2\text{-}F\text{-}G$

$A\text{-}B_9\text{-}C\text{-}D\text{-}E_3\text{-}F\text{-}G$

$A\text{-}B_{10}\text{-}C\text{-}D\text{-}E_4\text{-}F\text{-}G$

The general combinatorial formula for each library can be expressed as:

$$A_1\text{-}B_{10}\text{-}C_{10}\text{-}D_8\text{-}n(E_2)\text{-}F_1\text{-}G_1 \quad [III]$$

providing 1 x 10 x 10 x 8 x n x 2 x 1x 1 = 1600n compounds.

Both compound libraries, L1 and L2, of the above type are synthesized using solid phase techniques using the Multipin approach[9] such that each library contains 1600n compounds as 80n mixtures of 20 distinct, identifiable compounds. These 20 component mixtures are then placed separately into each of 80 wells of a 96 well plate (the other two lanes are used for control experiments) and then

libraries L1 and L2 (e.g. in the preferred embodiment: 1600n, where n = any integer between 1 and 4) the libraries themselves are complementary and amenable to deconvolution without recourse to resynthesis. It is also an important part of the invention that the library matrix has been especially formatted so that the most important site pairings P_1 and P_1' for proteolytic enzymes can be identified immediately without recourse to resynthesis.

Those compounds of the type A-B-C-D-E-F-G that are the better substrates for the protease will be cleaved, and can be readily identified because the fluorescor, A, will be cleaved from its nearby quencher F, in a time dependent manner which can be easily quantified. The fluorescent quenching by F of A only occurs when the two are in nearby proximity, normally within 30 angstrom units. Hence cleavage of a scissile bond (e.g. the scissile bond D-E) allows F to move further away from A and thus allow A to fluoresce when excited by light of the correct wavelength.

In this manner the most active compound can be rapidly identified without the need for further resynthesis and deconvolution. Moreover, the wells that show the most rapid development of fluorescence can also be analysed by mass spectrometry, since by comparison with the original mixture, the identity of the most efficient substrate can be found by its

Hence the problem of library deconvolution can be overcome and the most active substrate for the enzyme can be rapidly identified.

In addition, after the initial treatment of the proteolytic enzyme with the library mixtures, L1 and L2, the residual enzymatic activity in each well can be quantified by the addition of the most potent fluorogenic substrate for the enzyme, S1, which is found in the 16xn compound library. Because of the nature of the library design this can be quickly prepared and purified. If there is no appearance of increased fluorescence with the known substrate, S1, then the presence of an enzyme inhibitor can be inferred, which again can be quickly identified without the need for resynthesis.

The general description of the library layout will now be described with reference to figures 1 to 14.

For example, when n=1 and the library contains 1600 compounds, in the first column of the first row (A1) (Fig.1) in the first plate (P1) of the library L1, hereinafter designated as location A1,P1,L1 there will be one J component, J_1, one B component, B_1, the ten b components and the two E components (E_1 and E_2) (Fig. 2). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, hereinafter designated as

[illegible] in the first plate [illegible] of the library L1,

(hereinafter designated as location H10,P1,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_1 and E_2). Hence all 160 components are present in the one plate, because the 80 wells each contain 20 components.

A second complementary library is synthesised as follows (Fig. 3). In the first column of the first row (A1) of the first plate (P1) of the library, L2, (hereinafter designated as location A1,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_1, and one E component, E_1. In the tenth column of the first row (A10) of the first plate (P1) of the library, L2, (hereinafter designated as location A10,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the first plate (P1) of the library, L2, (hereinafter designated as location B1,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_1, and one E component, E_2. In the tenth column of the second row (B10) of the first plate (P1) of the library, L2, (B10,P1,L2) there will be ten C components, two D components (D_1 and D_2), one B component, B_{10}, and one E component, E_2. Hence only the first two rows are used to accommodate 40 compounds in total.

In the first column of the first row (A1) of the second

component, E_1 (Fig. 4). In the tenth column of the first row (A10) of the second plate (P2) of the library, L2, (hereinafter designated as location A10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the second plate (P2) of the library, L2, (hereinafter designated as location B1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1, and one E component, E_2. In the tenth column of the second row (B10) of the second plate (P2) of the library, L2, (B10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_2. Hence only the first two rows are used to accommodate 400 compounds in total.

In the first column of the first row (A1) of the third plate (P3) of the library, L2, (hereinafter designated as location A1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_1 (Fig 5). In the tenth column of the first row (A10) of the third plate (P3) of the library, L2, (hereinafter designated as location A10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the third plate (P3) of the library, L2, (hereinafter designated as location B1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E

D_7), one B component, B_{10}, and one E component, E_1. Hence only the first two rows are used to accommodate 400 compounds in total.

In the first column of the first row (A1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A1,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_1, and one E component, E_1 (Fig. 6). In the tenth column of the first row (A10) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A10,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location B1,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_1, and one E component, E_2. In the tenth column of the second row (B10) of the fourth plate (P4) of the library, L2, (B10,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_{10}, and one E component, E_2. Hence only the first two rows are used to accommodate 400 compounds in total.

In this fashion two complementary libraries, L1 and L2 are prepared. In library, L1, each of the 80 of wells contains a mixture of 20 components providing 1600 compounds for screening. In library, L2, four plates are

library L1, but in a format in which no two compounds found together in library, L1, will be found together in library, L2.

Thus it is an important part of the invention that the compounds contained in the two libraries L1 and L2 are themselves complementary, in that any two compounds which are found together in a 20 component mixture in the same location (e.g. A1P1L1) in library L1, are not found together in any of the 20 component mixtures in any location of the library L2.

Thus, for example, with reference to the primary library P1 L1 of figure 2 and the secondary libraries P1 L2, P2, L2, P3 L2 and P4 L2 of figures 3-6 it is possible to deconvolute an exemplary sequence:

$$-B_2-C_3-D_4-E_1-$$

If this sequence is a substrate fluorescence will occur in P1 L1 at C_3D_4. This gives the information that the substrate is

$$-?-C_3-D_4-?-$$

If fluorescence occurs in P2 L2 at B_2E_1 it indicates a

The confirmation of the substrate as

$$-B_1-C_1-D_4-E_1-$$

should be provided by non-fluorescence of P1 L2, P3 L2 and P4 L2 which all contain $-B_1-C_1-X-E_1-$ where X is not D_4.

In practice it is likely that more than one sequence will result in a substrate. Information as to which positions B-C-D-E- are sensitive to change (i.e. require a specific group) and which are insensitive (i.e. can tolerate more than one choice of group) in the context of the whole sequence gives valuable SAR data which can be used to model and/or synthesise related compounds.

In analogous examples, where separately n=2, 3 or 4, extra plates are constructed in library L1 format to accommodate the component pairs E_3 and E_4 (n =2), E_5 and E_6 (n = 3), and E_7 and E_8 (n = 4), respectively. For the respective deconvolution libraries of the type, L2, the respective rows in the plates P1, P2, P3, and P4, are increasingly filled with the paired components [illegible] and D_1, [illegible] and D_2, and [illegible] and D_3, respectively.

For example, when n = 3, and the library contains 4800 compounds, in the first column of the first row (A1 in the first plate (P1) of the library L1, (hereinafter

of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the first plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C_{10}, one D component, D_{10}, the ten B components and the two E components (E_1 and E_2). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the second plate (P2) of the library L1, (hereinafter designated as location A1,P2,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_3 and E_4). In the tenth column of the first row (A10) in the second plate (P2) of the library L1, (hereinafter designated as location A10,P2,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_3 and E_4). In the tenth column of the eighth row (H10) in the second plate (P2) of the library L1, (hereinafter designated as location H10,P2,L1) there will be one C component, C_{10}, one D component, D_{10}, the ten B components and the two E components (E_3 and E_4). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components.

location A1,P3,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_1 and E_2). In the tenth column of the first row (A10) in the third plate (P3) of the library L1, (hereinafter designated as location A10,P3,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the third plate (P3) of the library L1, (hereinafter designated as location H10,P3,L1) there will be one C component, C_{10}, one C component, C_1, the ten B components and the two E components (E_1 and E_2). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components. In total the three plate, P1, P2 and P3, contain 1600 compounds/plate 4800 compounds in total.

For example, when n = 4, and the library contains 6400 compounds, in the first column of the first row (A1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_1 and E_2) (Fig. 7). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the first plate (P1) of the library L1, (hereinafter designated as location

1600 components are present in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the second plate (P2) of the library L1, (hereinafter designated as location A1,P2,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_3 and E_4) (Fig. 8). In the tenth column of the first row (A10) in the second plate (P2) of the library L1, (hereinafter designated as location A10,P2,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_3 and E_4). In the tenth column of the eighth row (H10) in the second plate (P2) of the library L1, (hereinafter designated as location H10,P2,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_3 and E_4).

In the first column of the first row (A1) in the third plate (P3) of the library L1, (hereinafter designated as location A1,P3,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_3 and E_4) (Fig. 9). In the tenth column of the first row (A10) in the third plate (P3) of the library L1, (hereinafter designated as location A10,P3,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_3 and E_4). In the tenth column of the eighth row (H10) in the third plate

D component, D_1, the ten B components and the two E components (E_9 and E_{10}).

In the first column of the first row (A1) in the fourth plate (P4) of the library L1, (hereinafter designated as location A1,P4,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_9 and E_{10}) (Fig. 10). Likewise, in the tenth column of the first row (A10) in the fourth plate (P4) of the library L1, (hereinafter designated as location A10,P4,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_9 and E_{10}). In the tenth column of the eighth row (H10) in the fourth plate (P4) of the library L1, (hereinafter designated as location H10,P4,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_7 and E_8).

A second complementary library is synthesised as follows. In the first column of the first row (A1) of the first plate (P1) of the library, L2, (hereinafter designated as location A1,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_1, and one E component, E_1 (Fig. 11). In the tenth column of the first row (A10) of the first plate (P1) of the library, L2, (hereinafter designated as location A10,P1,L2), there will be the ten C components, two D components (D_1 and D_2), one B component, B_1, and one E component, E_1. In the first

components (D_3 and D_4), one B component, B_1, and one E component, E_4. In the tenth column of the eighth row (H10) of the first plate (P1) of the library, L2, (H10,P1,L2) there will be the ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_8. Hence the matrix containing all ten columns and all eight rows are used to accommodate 1600 compounds in total.

In the first column of the first row (A1) of the second plate (P2) of the library, L2, (hereinafter designated as location A1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1, and one E component, E_1 (Fig. 12). In the tenth column of the first row (A10) of the second plate (P2) of the library, L2, (hereinafter designated as location A10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the second plate (P2) of the library, L2, (hereinafter designated as location B1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1, and one E component, E_2. In the tenth column of the eighth row (H10) of the second plate (P2) of the library, L2, (H10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_8.

In the first column of the first row (A1) of the third plate (P3) of the library, L2, (hereinafter designated as

row (A10) of the third plate (P3) of the library, L2, (hereinafter designated as location A10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the third plate (P3) of the library, L2, (hereinafter designated as location B1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_1. In the tenth column of the eighth row (H10) of the third plate (P3) of the library, L2, (H10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_1.

In the first column of the first row (A1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A1,P4,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_1 (Fig.14). In the tenth column of the first row (A10) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A10,P4,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location B1,P4,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_1. In the tenth column of the eighth row (H10) of the fourth plate (P4) of the library, L2, (H10,P4,L2), there will be ten C components, two D components

The FRET strategy is based on the synthesis of two orthogonal sets of mixtures in solution. These solutions are each indexed in two dimensions. Thus the data from, for example, a protease scan identifies the most active compounds without the need for decoding or resynthesis. The positional preferences of sub-units (in this case amino acids) are optimised with respect to all other variant positions simultaneously. The synergistic relationship between all four positions is realised and both positive, beneficial and negative, deactivating data are generated. This leads to families (sub-populations) of substrates and their sub-unit preferences. The data can be fed into molecular modelling programs to generate pharmacophoric descriptors that encompass both the desirable features (from the positive data) and indicate undesirable interactions (from the negative data sets). Note that a one dimensional scan only indicates one position at a time as 'most active' and does not explore the synergistic relationship between positions.

The general methodology exemplified above with regard to the use of complementary combinatorial FRET libraries for the identification of proteolytic enzyme substrates, is equally applicable for identification of compounds from a library which interact with another active moiety. Combinatorial libraries of compounds containing four variable groups B, C, D and E can be produced an interactions with active moieties detected using suitable reporters or markers.

For example, the active moiety may be a protein kinase. Protein kinases include (but are not limited to) the following:

ZAP-70, Syk, p56 ^{lck}, p59^{fyn}, Yes, Hck, Src, Btk, Blk, Lyn, Raf, EGF kinase, Insulin receptor kinase, cyclin dependent kinases, Bcl-Abl, IkB-kinase C-terminal kinases, IkB N-terminal kinases, Jak kinases, MAP kinase kinases, MAP kinase kinase kinases, MAP kinases (Erks 1-3, p38 and Jun kinases), STAT family C-terminal serine/threonine kinases, protein kinase A, protein kinase B, all protein kinase C isoforms.

The active interaction of a protein kinase with a compound in a library may be detected, for example, by radioactively labelled phosphorylation of the substrate in the library.

Thus it is possible to identify peptide substrates of protein tyrosine kinase by phosphorylation of exogenous peptide substrates present in a library. The assay uses the transfer of ^{33}P from radiolabelled ATP to lysine-tagged tyrosine-peptides substrate as a measure of kinase activity. The peptide binds to negatively charged membrane and is washed free of unincorporated [γ-^{33}P]-ATP. The detection of β-energy emission using a fluor-containing scintillant on a β-counter instrument provides the measurement of peptide phosphorylation.

In another example the active moiety could be a glycosidase and the interaction would be with enzyme substrates in the library. In such a case a scissile bond between at least one of the groups B, C, D and E would be an acetal or glycosidic bond. The groups B, C, D and E could be selected from furanosides and pyranosides.

In another example the active moiety could be a nuclease. In such a case the scissile bond would be a phosphodiester bond and the groups B, C, D and E would be nucleotides.

The general methodology of the present invention can also be applied to the detection of an interaction between an active moiety such as a receptor and a library containing ligands for the receptor. Examples of receptor/ligand interactions are shown by way of non-limiting illustration below:

Chemokine receptor/chemokine i.e. CKR3/Eotaxin

SH3 domain/proline amino acid sequence i.e. Grb2/SOS peptide

WW domain/proline amino acid sequence i.e. Yap (Yes-associated protein)/RSV Gag protein

SH2 domain/phosphopeptide i.e. Grb2/LNK phosphopeptide

Lectin domain/ligand i.e. CD72/CD5.

The reporter or marker for detection of a reaction in receptor/ligand and antibody/antigen libraries could be similar. Suitable reporters are, for example, (but not limited to):

1. Biotin tag on the ligand using streptavidin-HRP for detection,

2. Alkaline phosphatase tag,

3. Radioisotope tag.

The invention will now be described by reference to the following examples.

Example 1: Cysteine Protease Inhibitors and Substrates

In this Example the proteolytic enzyme of interest is Der P1, which is found in house dust mite faeces. The example illustrates the synthesis of a number of FRET compounds in which the suitable bond is an unsubstituted amide bond, their use as a library for screening for potential substrates of Der P1, and subsequent identification and synthesis of active inhibitors of the enzyme.

Purification of *Der pI*.

potassium phosphate; pH 7.4 containing 150 mM NaCl *Der pI* was purified by affinity column chromatography using 4C1 antibody (Indoor Biotechnology, Deeside, U.K.) The crude preparation was mixed with ~2 mL of affinity resin for 2 h at 4°C and then washed with 2-3 volumes of PBS. Elution of bound protein was carried out using 5mM glycine containing 50%(v/v) ethylene glycol. Fractions(2.2 mL) were collected and neutralised with 0.8 mL of 0.2 M sodium phosphate buffer, pH 7.0. The fractions were pooled and dialysed overnight against 4 L PBS followed by a second dialysis against 2 L PBS for 2-3h. The total protein was concentrated as required by ultrafiltration (MacroSep; Flowgen, U.K.)

Synthesis of compounds

The compounds were synthesised using the Multipin approach[25,26] using Fmoc-Rink amide Macro crowns (Chiron Mimotypes Pty., Ltd., 11 Duerdin Street, Clayton Victoria 3168, Australia) with a loading of 7 μMoles.

The amino acid residues of each of the compounds were linked using amide bonds in a suitable form. The coupling chemistry employed is similar to that reported in the literature[27] for fluorenylmethoxycarbonyl protected amino acids and activated pentafluorophenyl esters, in which the side-chains are protected using acid labile protecting groups known to those skilled in the art [illegible]

of Aspartic acid and Glutamic acid, Trityl (for the Amide of Asparagine and Glutamine, and the amine functionality of the Histidine ring.

The N-α-fluorenylmethoxycarbonyl protecting group of the coupled residues were cleaved using 20% piperidine in dimethylformamide (DMF) for 30 minutes at 20° C. The coupling reactions for the free acids such as Boc-ABz-OH (Boc-2-aminobenzoic acid), and Fmoc-(3-nitro)tyrosine-OH were accomplished using 10 equivalents of a mixture of the free acid (1 eq.) :TBTU (0.98 eq.): HOBt (0.98 eq.) : N-methylmorpholine (1.96 eq.) in dimethylformamide (500 μL) as solvent for 5 hours at 20° C. The other amino acids were coupled as their pentafluorophenyl esters[56] for 2-6 hours.

Hence, in order to couple approximately equal ratios of each component in the mixture of the derivatised amino acids as their pentafluorophenyl esters, a solution of a total of 0.98 equivalents (relative to the amino group loading on the crown) of the mixture of amino acid pentafluorophenyl esters : HOBT (1 eq.) in DMF (500 μL) were coupled for 16 hours at 20 °C. The pins were then washed well with DMF and then recoupled using the same mixture under the same conditions. A third coupling of 1 equivalents (relative to the amino group loading of the crown) for 2 hours in DMF was performed using this coupling protocol with equimolar mixtures of the derivatised pentafluorophenyl esters of the amino acids in

20 compounds present on each crown. The compounds were cleaved from the crowns directly into the 80 designated wells of the desired 96 well plate. In the cleavage protocol each crown was treated with a mixture (600 μL) containing trifluoroacetic acid (95%), triethylsilane (5%) for 2 hours at 20° C. The crowns were then washed with trifluoroacetic acid (500 μL) and this was then combined with the cleavage solution.

The Fmoc-Rink amide Macro crowns (Chiron Mimotypes Pty., Ltd., 11 Duerdin Street, Clayton Victoria 3168, Australia) at 7 μMol loading per crown, were coupled with a 10 fold excess of a mixture containing L-Fmoc-Asp(O-t-Bu)-OH (1eq) using TBTU (0.98 eq) and N-methylmorpholine (1.96 eq.) in the presence of HOBt (0.98 eq.) in DMF at 0.14M concentration. After deblocking of the Fmoc group with 20% piperidine in DMF for 30 minutes and subsequent washing with DMF and then methanol, coupling of the Fmoc-(3-nitro)tyrosine-OH was accomplished using 10 equivalents of a mixture of the Fmoc-(3-nitro)tyrosine-OH (1 eq.) :TBTU (0.98 eq.): HOBt (0.98 eq.) : N-methylmorpholine (1.96 eq.) in dimethylformamide as solvent at 0.14 M concentration for 5 hours at 20° C. Removal of the Fmoc group (*vide infra*) was followed by coupling of the mixtures of amino acids in the ratios outlined and under the conditions described (*vide infra*).

In a particular example the amino acids comprising group B

comprising group D include Ala, Val, Ile, Leu, Nle, Ser, Glu, Phe. For n=4, the amino acids comprising group E include Ala, Val, Ile, Leu, Nle, Ser, Glu, Phe. Otherwise any selection from the amino acids can be made for n=1, 2, or 3.

The plates containing the combined cleavage solutions and were then evaporated to dryness to yield the component mixtures using a rotary centrifuge ("SPEEDVAC", Savant Instruments Inc., Farmingdale, NY) at 800 rpm for 1 hour at 20° C under a reduced pressure of 10^{-1} mmHg. Each component was then transferred to the final mother plate using a (50%: 45%: 5%) mixture of acetonitrile: water: acetic acid. The plates were then lyophilised to dryness using at 20° C under a reduced pressure of 10^{-1} mmHg, and then stored at -20° C. In this fashion libraries of the type shown in Figures 2-14 were prepared.

In further detail, the Multipin approach which was employed is described below:

Multipin Synthesis Of Potential Substrates of *Der pI*

The 'Chiron' multipin kit consists of a standard 8 x 12 pin holder containing 96 'pin stems' to which are reversibly attached 'crowns'. The 'crowns' provide a reactive polymer surface upon which a growing peptide is

reactor by performing simultaneous synthesis in individual 1mL wells of industry standard 96 well plates. Each well, and thus each crown, can be charged with a unique set of reagents providing unique sequences to each crown. Common steps such as washing or removal of Nα protection can be performed concomitantly.

Synthesis is based upon the use of Nα-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids. Side-chains of tri-functional amino acids are protected with acid labile groups such as trityl or tert-butyl. The addition of amino-acid residues to the growing peptide chain, a process termed 'coupling' proceeds through the utilisation of pre-formed pentafluorophenyl (pfp) esters or activation of the free acid, using the reagents HBTU or BOP in the presence of tertiary base (NMM) and HOBt as catalyst.

The experimental techniques used are fully documented (Maeji, N. J. Bray, A. M. Valerio, R. M. and Wang, W., *Peptide Research*, **8(1)**, 33-38, 1995 and Valerio, R. M. Bray, A. M. and Maeji, N. J. Int. J. Pept. Prot. Res, **44**, 158-165, 1994) and the main steps are briefly as follows.

General Methods

Preparation of Multipin Assembly

onto stems and slotted into the 8 x 12 stem holder in the desired pattern for synthesis.

Removal of Nα-Fmoc Protection

A 250mL solvent resistant bath is charged with 200 ml of a 20% piperidine/DMF solution. The multipin assembly is added and deprotection allowed to proceed for 30 minutes. The assembly is then removed and excess solvent removed by brief shaking. The assembly is then washed consecutively with (200mL each), DMF (5mins) and MeOH (5mins, 2mins, 2mins) and left to air dry for 15mins.

Quantitative UV Measurement of Fmoc Chromophore Release

A 1cm path length UV cell is charged with 1.2mL of a 20% piperidine/DMF solution and used to zero the absorbance of the UV spectrometer at a wavelength of 290nm. A UV standard is then prepared consisting of 5.0mg Fmoc-Asp(OBut)-Pepsyn KA (0.08mmol/g) in 3.2mL of a 20% piperidine/DMF solution. This standard gives Abs_{290} = 0.55-0.65 (at RT). An aliquot of the multipin deprotection solution is then diluted as appropriate to give a theoretical Abs_{290} = 0.4, and this value compared with the actual experimentally measured absorbance showing the efficiency of previous coupling reaction.

Whilst the multipin assembly is drying, the appropriate Nα-Fmoc amino acid pfp esters (10 equivalents calculated from the loading of each crown) and HOBt (10 equivalents) required for the particular round of coupling are accurately weighed into suitable containers. Alternatively, the appropriate Nα-Fmoc amino acids (10 equivalents calculated from the loading of each crown), desired coupling agent e.g. HBTU (9.9 equivalents calculated from the loading of each crown) and activation eg HOBt (9.9 equivalents calculated from the loading of each crown), NMM (19.9 equivalents calculated from the loading of each crown) are accurately weighed into suitable containers.

The protected and activated Fmoc amino acid derivatives are then dissolved in DMF (500μl for each macrocrown, e.g. for 20 macrocrowns, 20 x 10eq x 7mmoles of derivative would be dissolved in 10 000μL DMF). The appropriate derivatives are then dispensed to the appropriate wells ready for commencement of the 'coupling cycle'. As a standard, coupling reactions are allowed to proceed for 2-6 hours (depending upon nature of coupling e.g. Ala to Ala 2 hours Val to Leu 6 hours.

When coupling Fmoc amino-acid pentafluorophenyl esters, 10eq of derivative in DMF (400μl) with bromophenol blue stock solution (100μl) is used for each macrocrown. This allows monitoring of the progress of the acylation

unreacted amine to a pale yellow upon completion of acylation.

Preparation of Bromophenol Blue Stock Solution

Bromophenol blue (20mg) is dissolved in DMF (50mL) and HOBt (10mg) added.

Washing Following Coupling

If a 20% piperidine/DMF deprotection is to immediately follow the coupling cycle, then the multipin assembly is briefly shaken to remove excess solvent washed consecutively with (200mL each), MeOH (5mins) and DMF (5mins) and deprotected (see above). If the multipin assembly is to be stored, then a full washing cycle consisting brief shaking then consecutive washes with (200mL each), DMF (5mins) and MeOH (5mins, 2mins, 2mins) is performed.

Acidolytic Mediated Cleavage of Peptide-Pin Assembly

Acid mediated cleavage protocols are strictly performed in a fume hood. A polystyrene 96 well plate (1mL / well) is labelled, then the tare weight measured to the nearest mg. Appropriate wells are then charged with a trifluoroacetic

The multipin assembly is added, the entire construct covered in tin foil and left for 2hrs. The multipin assembly in then added to another polystyrene 96 well plate (1mL /well) containing trifluoroacetic acid / triethylsilane (95:5, v/v, 600µl) (as above) for 5 mins.

The cleaved assembly is washed with DMF (200µL, 5mins), MeOH (200µL, 5mins), the spent crowns removed and discarded, the stems removed and washed by sonication in methanol (1hr, RT).

Work up of Cleaved Peptides

The primary polystyrene cleavage plate (2hr cleavage) and the secondary polystyrene plate (5min wash) (see above) are then placed in the SpeedVac and the solvents removed (minimum drying rate) for 90mins.

The contents of the secondary polystyrene plate (see above) are transferred to their corresponding wells on the primary plate using an acetonitrile / water / acetic acid (50:45:5, v/v/v solution (3 x 150µl) and the spent secondary plate discarded

Analysis of Products

0.1%aq trifluoroacetic acid, Solvent B = acetonitrile / 10%A. Gradient :- 10-90% B in A over 27mins, 250ml / min, 215nm UV detection. The individual substrates described below were prepared by the above methods and shown by HPLC-MS to be >95% with the correct mass.

Final Lyophilisation of Peptides

The primary polystyrene plate (plus the washings from the secondary plate) is covered with tin foil, held to the plate with an elastic band. A pin prick is placed in the foil directly above each well and the plate placed at -80°C for 30mins. The plate is then lyophilised on the 'Heto freeze drier' overnight. Where appropriate individual peptides were then weighed and dissolved to 10mM stock solutions in DMSO prior to biological screening. Alternatively the 20 component mixture is weighed and the peptide/20 component ratio is calculated.

Further coupling of amino acid residues was carried out according to the multipin approach described above. Whilst the multipin assembly was drying, the appropriate Nα-Fmoc amino acids (9.9 equivalents calculated from the loading of each crown), HATU coupling agent (9.9 equivalents calculated from the loading of each crown), HOAt catalyst (9.9 equivalents calculated from the loading of each crown) and DIPEA (19.8 equivalents calculated from the

The protected Nα-Fmoc amino acids and coupling agents were then dissolved in DMF (500 µl for each macrocrown) and activated by the addition of DIPEA. The appropriate derivatives were then dipensed to their appropriate wells and as standard coupling to each macro crown was allowed to proceed for 2 hours.

When coupling particularly hindered amino acid residues such as N-Methyl, Cα-Methyl or unusual amino acids (whose coupling efficiency is unknown) the coupling reaction was repeated, as standard, for a further 2 hours.

Substrates for *Der pI*

Using the general techniques described above, the following compounds were prepared and assayed as potential substrates against *Der pI* purified as described above.

Peptide [SEQ ID Nos. 1-76]	Measured K_m (µM)
Abz-Val-Ala-Nle-Ser-Tyr(NO$_2$)-Asp-NH$_2$	12
H-Val-Ala-Nle-Ser-Tyr(NO$_2$)-Asp-NH$_2$	NS
H-Ala-Nle-Ser-Tyr(NO$_2$)-Asp-NH$_2$	NS
Ac-Val-Ala-Nle-Ser-Tyr(NO$_2$)-Asp-NH$_2$	NS
H-Val-Ala-Nle-Ser-Tyr(NO$_2$)-NH$_2$	NS
[illegible]	[illegible]

Abz-Val-Ala-Nle-Ser-NH_2	NM
Abz-Val-Ala-Nle-Ser-Phe-Asp-NH_2	NM
Abz-Val-Ala-Nle-Ser-Tyr-Asp-NH_2	NM
Abz-Val-Ala-Nle-Ser-Ala-Asp-NH_2	NM
Abz-Val-Ala-Nle-Ser-Lys-Asp-NH_2	NM
Abz-Val-Ala-Nle-Ser-eAHA-Asp-NH_2	NM
Abz-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Bz-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[a]
Bz(2-carboxy)-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[a]
Chex-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[a]
n-Bu-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[a]
Piv-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[a]
Bz-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[a]
Abz-Val-Ala-Lys-Ser-Tyr(NO_2)-Asp-NH_2	14
Abz-Val-Ala-Gln-Ser-Tyr(NO_2)-Asp-NH_2	6
Abz-Val-Ala-Thr-Ser-Tyr(NO_2)-Asp-NH_2	6
Abz-Val-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	4
Abz-Val-Ala-Cha-Ser-Tyr(NO_2)-Asp-NH_2	5
Abz-Val-Ala-His-Ser-Tyr(NO_2)-Asp-NH_2	> 21
Abz-Val-Ala-AOH-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Ala-DNle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Ala-3pyr-Ser-Tyr(NO_2)-Asp-NH_2	10
Abz-Val-Ala-Hyp-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-[illegible]-Tyr(NO_2)-Asp-NH_2	N

Abz-Val-Tic-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-ACH-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Met(O)-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	35
Abz-Val-2Nal-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-ACP-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-DLys-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-DGln-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-3pyr-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Cha-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-DVal-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Gln-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	12
Abz-Lys-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	>15
Abz-Tic-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-ACH-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Met(O)-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	20
Abz-3pyr-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	>10
Abz-2Nal-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	15
Abz-Leu-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	18
Abz-Cha-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	[illegible]
Abz-Bip-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	2.5
Abz-Bip-Ala-hLeu-Tyr-Tyr(NO_2)-Asp-NH_2	[illegible]
Abz-Bip-Ala-hLeu-Leu-Tyr(NO_2)-Asp-NH_2	3.7
Abz-Bip-Ala-hLeu-Lys-Tyr(NO_2)-Asp-NH_2	2
Abz-Bip-Ala-hLeu-Asp-Tyr(NO_2)-Asp-NH_2	9.0
Abz-Bip-Ala-hLeu-Met-Tyr(NO_2)-Asp-NH_2	[illegible]

Abz-Bip-Ala-hLeu-Thr-Tyr(NO_2)-Asp-NH_2	2.5
Abz-Bip-Ala-hLeu-3pyr-Tyr(NO_2)-Asp-NH_2	4
Abz-Bip-Ala-hLeu-ButGly-Tyr(NO_2)-Asp-NH_2	4
Abz-Bip-Ala-hLeu-Hyp-Tyr(NO_2)-Asp-NH_2	4
Abz-Phe-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-3.Pyr-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-1.Naph-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	17
Abz-2.Naph-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-Tyr-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-Bip-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	10
Abz-Lys-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	15
Abz-Glu-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	20
Abz-Leu-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-Hyp-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS

NS indicates that the peptide was not hydrolysed by *Der pI*.

NM indicates that the peptide was a substrate for *Der pI*, but the K_m was not measured.

NM' indicates that the peptide was a substrate for *Der pI*, and its cleavage was followed by HPLC-MS showing hydrolysis to occur between-Nle-Ser-.

Ranked in order of cleavage rate :-Bz > n-But > Piv > Bz(2-carboxy) > Abz.

Coupling of amino acid residues was carried out according to the multipin approach described above. Whilst the multipin assembly was drying, the appropriate Nα-Fmoc amino acids (10 equivalents calculated from the loading of each crown), HATU coupling agent (9.9 equivalents calculated from the loading of each crown), HOAt catalyst (9.9 equivalents calculated from the loading of each crown) and DIPEA (19.9 equivalents calculated from the loading of each crown) were accurately weighed into suitable containers.

The protected Nα-Fmoc amino acids and coupling agents were then dissolved in DMF (500 µl for each macrocrown) and activated by the addition of DIPEA. The appropriate derivatives were then dispensed to their appropriate wells and standard coupling to each macrocrown was allowed to proceed for 2 hours.

When coupling particularly hindered amino acid residues such as N-Methyl, Cα-Methyl or unusual amino acids (whose coupling efficiency is unknown) the coupling reaction was repeated, as standard, for a further 2 hours.

The following sequences were synthesised in this way:

Peptide Sequence (SEQ ID Nos. 77-85) Measured Km(µM)

Abz-Val-Ala-Aib-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Aib-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Deg-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
nPu-D.Ser-D.Nle-D.Ala-D.Val-p.Aba-NH_2	NS
Bz-Val-Ala-Statine-Ser-eAha-NH_2	NS
Abz-p.Aba-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Cmpi-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS

NS-Not Substrate: Not hydrolysed by *Der pI*

NM-Substrate but not measured: Substrate for *Der pI* but not measured.

Assay procedure

Each mixture of 20 compounds in the libraries of the apparatus described herein was screened at a concentration of 1.0 μM per compound in an assay against the cysteinyl protease *Der pI*. The most active wells were identified by the rate of emission of fluorescence at 420 nm when the samples were irradiated at 320 nm. An analysis of the two complementary libraries showed that the best substrates for the enzyme were:

Abz-B-C-D-E-Tyr(NO2)-Asp-NH2

Where

E=Serine

The best substrate was:

[SEQ ID:1] Abz-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2

This compound was then resynthesised as a single component using the peptide synthesis methodology described herein. The k_{cat}/K_m value for the pure substrate in the *Der pI* assay was measured as $3.5 \times 10^4 M^{-1}s^{-1}$, and was considered to be suitably high for use in a high throughput assay for the general screening of inhibitors of *Der pI*.

High throughput assay development

Plate assays were carried out in 96 well plate format, using 0.1 ug of *Der pI* per 100 µL assay volume in each well and using 20 µM of the substrate. All assays were performed in Assay Buffer (AB; 50 mM potassium phosphate, pH 8.25 containing 1mM ethylenediaminetetraacetic acid (EDTA) and 1mM dithiothreitol (DTT). The Der pI enzyme is pre-activated by addition of DTT and this in incubated at room temperature for 5 min. prior to initiation of the assay. As an example for the screening methodology, each well contains a 5 µL of a 20 µM solution of the test compound in DMSO, 10 µL of a 200 µM aqueous solution of the substrate, [illegible] and 85 µL of [illegible] pI in AB is added [illegible]

machine. Kinetic measurements were carried out using a Hitachi F-4500 Fluorescence Spectrophotometer.

Synthesis of inhibitors of *Der pI*

The best substrate described above was shown by HPLC-mass spectroscopic analysis of the enzyme/ assay solution, to be cleaved between the Norleucine-Serine amide bond. Replacement of the terminal Abz group by a series of derivatives (e.g. Boc-, Pivaloyl, Benzoyl, and 2- carboxy-Benzoyl) affected substrate activity and specificity for the *Der pI* enzyme. With this knowledge of the P_1-P_1' cleavage site and for the P_4-P_3-P_2-P_1 motif, the compound Boc-Val-Ala-Leu-H, 4, was synthesised as shown in Scheme 1a, figure 15.

Attachment of a suitable Michael acceptor such as CH=CH-CO2Et, and -CH=CH-SO_2 Ph to the motif (Scheme 2, figure 16), provided active inhibitors of the enzyme with apparent IC_{50} values of 50nM, 1000 nM and 100nM respectively.

Based on the sequence 4, a series of acyloxymethylketone compounds having active Der PI inhibitor activity was prepared. Details of the preparation and activity of this novel group of cysteine protease inhibitor compounds are discussed in co-pending International Application

Example 2: Viral Protease Inhibitors and Substrates

Design of Depsipeptides

Another suitable bond in a compound of general formula (I), (II) or (III) according to the invention is an ester bond to form a depsipeptide. The incorporation of depsipeptide substrates aided the identification of substrates for low reactivity viral proteases, such as viral serine proteases.

For example, substrates of the general formula

n [Abz-$B_{1,6}$-$C_{1,6}$-$D_{1,6}$y[CCO]-$E_{1,6}$-Tyr(NO_2)-Asp-NH_2]

were produced.

However, a significant proportion of viral proteases only recognise substrate sequences larger than those represented by the general structure above. It is well acknowledged that by the very nature of action of a viral protease (function is to cleave immature viral proteins into the mature viral package one automatically receives data concerning the natural substrate sites. Thus, the general structure above can be extended by introducing extra fixed amino-acids at appropriate sites. A logical extension would be to introduce the known P1-P1' cleavage site as the depsipeptide bond, then subsequently introduce the four variant positions following the standard format

n [Abz-B_{1-10}-C_{1-10}-D_{1-8}-E_{1-4}-P1 y[COO]-P1'-Tyr(NO_2)-Asp-NH_2]

Furthermore, if these substrates again proved to be too small, one may use the known substrate sequences to introduce additional fixed positions. For instance, with Hepatitis NS3 protease it is known that the natural P6 position is a conserved acidic residue (aspartic or glutamic acid). Thus one could extend the above structure as detailed below.

n [Abz-P6-B_{1-10}-C_{1-10}-D_{1-4}-E_{1-8}-P1 y[COO]-P1'-Tyr(NO_2)-Asp-NH_2]

The novel methodology described herein greatly faciltiates the invention of therapeutically useful proteolytic enzyme inhibitors and is commercially exploitable. This is because the best substrate motif for the proteolytic enzyme can be rapidly identified, and, since there exist in the literature a variety of ways for attaching motifs which react with the active site of a proteolytic enzyme, especially for aspartyl, metallo, serine and cysteinyl proteases, an enzyme inhibitor can be readily synthesised. Moreover, amide bond replacements or transition state mimetics can be incorporated into the molecule, which would be especially useful for the inhibition of aspartyl or metallo proteases.

The method described also facilitates the rapid

for the detection of inhibitors of the particular proteolytic enzyme under scrutiny.

The presence of an inhibitor within the compound libraries described is readily detected by retreatment of the assay mixture with the most active fluorogenic substrate, which will allow the immediate measurement of the remaining proteolytic enzyme activity.

The invention provides self-decoding, combinatorial fluorogenic libraries, and it will greatly facilitate the design and invention of novel protease inhibitors because:

i. The peptides of the library may have increased aqueous solubility in comparison to peptides containing similar and other fluorogenic and quencher groups.

ii. The peptides are stable to contaminating exopeptidases.

iii. The self deconvolution method described, coupled with the continuous analysis of the rate of substrate cleavage data, allows the immediate identification of the most active binding motif contained within the substrate library.

iv. The method allows for the rapid assessment of the enzyme assay mixture for any

Example 3: Kinase Inhibitors and Substrates

As previously indicated, the present invention is a broadly applicable methodology and is not limited to the use of FRET libraries to detect protease inhibitor/substrate compounds.

An example of another use of the invention is the detection of the interaction of a kinase with a substrate/inhibitor by monitoring radioactively labelled phosphorylation of compounds of complementary combinatorial libraries.

The Identification of a ZAP-70 Tyrosine Kinase Inhibitor

ZAP-70 is a intracellular kinase essential for T-cell signal transduction from the T-cell antigen receptor. Mutations resulting in non-functional ZAP-70 kinase have been observed in patients suffering from a severe combined immunodeficiency disease. Drug down-modulation of ZAP-70 kinase activity has therefore been seen by the major pharmaceutical companies as a potential method of down regulating the immune system in an antigen-independent manner. Such a drug could be used in both transplant treatment and as a therapy in autoimmune diseases.

Generation of ZAP-70 kinase catalytic domain

recruitment to phosphorylated tyrosine residues on the T cell antigen receptor, but are unnecessary for protein kinase catalytic activity. Removal of the SH2 domains results in a constitutively active, stable ZAP-70 kinase. Specific oligonucleotide polymerase chain reaction (PCR) primers were used to amplify amino acids 308 to 619 and add a carboxy-terminal hexahistidine sequence using human Jurkat cDNA as a template. This amplicon was cloned into the baculoviral transfer vector pACUW51 (Pharmingen) and recombinant baculovirus produced by homologous recombination in Sf9 insect cells.

CatZAP(H6) Purification

Low titre stocks of mixed CatZAP-70 baculovirus were used to infect sub-confluent monolayers of Sf9 cells. Cells were harvested 3 days post-infection, washed in PBS and osmotic lysis performed immediately. Isolation of CatZAP(H_6) protein was carried out using cobalt column chelation chromatography, fractions eluted using 100 mM imidazole and kinase activity determined by ^{32}P transfer.

High throughput protein kinase screening assay

The interaction of the active protein kinase moiety with compounds of a combinatorial peptide library can be determined.

produced by CatZAP(H_s). The peptide library was synthesised with a positively charged tetra-lysine tag. After peptide incubation with the protein kinase and ATP, containing trace amounts of radiolabelled [^{33}P-γ]-ATP, the reaction mixture is passed through a negatively charged phosphocellulose membrane using a 96 well plate vacuum manifold system (Millipore). Positively charged peptide is bound by the membrane, whereas unincorporated [33P-γ]-ATP is washed through. Scintillant is then applied to the membrane and the amount of phosphorylated peptide calculated from the level of radioactivity on the membrane, measured using a well plate scintillation counter. This type of assay can be readily applied to other protein kinase systems.

The result of this screening assay is the identification of one or more protein kinase substrate motifs, which appear to have the common features of a negatively charged N-terminus and a conserved Pro at the C-terminus.

For other protein tyrosine kinases, the existing libraries may provide some information, although it is envisaged that additional libraries will require to be built. For Ser/Thr kinases, the technology may be adapted by the use of a central Ser or Thr residue as the phosphoacceptor moiety.

In addition, technology is being developed within the chemistry department which will allow for the generation

Abbreviations

Abbreviations used herein are as follows:

Abbreviations for amino acids and nomenclature of peptide structures follow the recommendations given in: IUPAC-IUB Commission on Biochemical Nomenclature, (*J. Biol. Chem*, **247**, 997, 1971). All chiral amino acids are of the L configuration unless otherwise stated. Other abbreviations used are :

-Abu , b-amino butyric acid, : Abz, 2-amino benzoyl : ACH, 1-amino-1-carboxy-cyclohexane : ACP, 1-amino-1-carboxy-cyclopropane : Bip, Biphenylalanine : n-Bu, n-butoxycarbonyl : Bz, Benzoyl : Bz(2-carboxy), 2-carboxybenzoyl : But-Gly, tert-Butylglycyl : BOP, benzotriazoyl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate: Cha, cyclohexylalanine : Chex, 1-carboxycyclohexyl : eAHA, gamma aminohexanoyl : HBTU, O-benzotriazoyl-N,N,N',N'-tetramethyluronium hexafluorophosphate : HOBt, 1-hydroxybenzotriazole : Hyp, trans-4-hydroxyprolinyl : hLeu, homoleucyl : 2Nal, 2-napthylalanine : NMM, N-methylmorpholine : Pic, picoyl : 3pyr, 3-pyridylalanine : Tic, 1-carboxytetrahydroquinolyl : Tyr(NO$_2$), 3-nitrotyrosine.

DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl;

chroman, Pbf, pentamethylbenzofuran, TBTU, 2-(1H-Benztrotriazole-1-yl)-1,1,1,3,3-tetramethyluronium tetrafluoroborate; Trt, Trityl.

p.Aba, 4-aminobenzoyl; Aib, Aminoisobutyric acid; Bip, Biphenylalanine; nBu, n-Butyl; Bz, Benzoyl; Cmpi, Carboxymethylpiperazine; Deg, Diethylglycine; DIPEA, N,N-Diisopropyl-ethylamine; HATU, O-(7-azabezotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAT, 1-hydroxy-7-azabenzotriazole; Naph, Naphthylalanine; 3.Pyr, 3-pyridiylalanine; Tyr(NO$_2$), 3-nitro-tyrosine.

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Claims

1. Libraries of compounds which interact with an active moiety, the libraries comprising two orthogonal sets of mixtures of compounds in solution providing two complementary combinatorial libraries (referred to as primary and secondary libraries) indexed in two dimensions for autodeconvolution, thereby allowing characterisation of an active motif in any group of compounds.

2. A complementary pair of compound libraries L1 and L2 according to claim 1 which constitute a set containing compounds having four variable groups B, C, D, E of formula:

-Bb-Cc-Dd-n(Ee)-

giving b x c x d x e = Mn compounds in each library, there being a predetermined number (P1, P2) of mixtures each consisting of a predetermined number (Q1, Q2) of individual identifiable compounds in each library, wherein both L1 and L2 contain the same Mn compounds, but wherein any two compounds which are found together in one mixture of Q1 compounds of L1 are not found together in any one of the P2 mixtures of L2.

3. A method of screening for interaction of an active

mixtures of L2 are each placed separately into individual wells of well plates, the well plates having wells arranged in a format adapted to allow deduction of a unique active compound formula from the presence of activity in one well of L1 and one well of L2.

4. A method according to claim 3 wherein the format complies with general deconvolution formulae in which:

(i) $ns = \frac{Rp.Cp.}{Rs.Cs} Np$

(ii) $k = b.c.d.np.e$

(iii) $k = x.N.np$

(iv) $N = Rp.Cp$

(v) $K = X.Rp.Cp.np$

(vi) $b.c.d.e = X.Rp.Cp$

(vii) $Cp.e = X$

(viii) [illegible]

np = number of primary plates

ns = number of secondary plates

Rp = number of primary rows

Rs = number of secondary rows

Cp = number of primary columns

Cs = number of secondary columns

K = number of combinations of compounds

N = number of wells on a plate, and

X = number of compounds per well.

5. A method according to claim 4 wherein

np = 4

ns = 16

Rp = 8

Rs = 4

Cp = 10

Cs = 5

K = 6400

N = 80

X = 20

6. A method according to claim 5 wherein the active moiety is selected from the group consisting of enzymes,

7. A method according to claim 6 wherein interaction of the active moiety with the compounds of the library is selected from the group consisting of the interaction of a substrate or inhibitor with an enzyme, the interaction of a ligand with a receptor, and the interaction of an antigen or antigenic epitope with an antibody.

8. A method according to claim 6 wherein the active moiety is selected from the group consisting of the following enzyme classes:

1. Oxidoreductase

a) dehydrogenase

b) oxidase

c) peroxidase

d) catalase

2. Hydrolase

f) peptidase (proteolytic enzyme)

3. Transferase

g) aminotransferase

h) kinase

i) glucosyltransferase

5. Isomerase

l) racemase

m) mutase

6. Ligase

n) synthetase

o) carboxylase

9. A method according to claim 8 wherein the active moiety is a protease selected from the group consisting of the following:

1. Aspartyl proteases, such as renin, HIV, cathepsin D and cathespin E.

2. Metalloproteases, such as ECE, gelatinase A and B, collagenases, stromolysins.

3. Cysteinyl proteases, such as papain, ICI, CerP1, cathepsin B, cathepsin K.

4. Serine proteases, such as thrombin, factor VIIa, factor Xa, elastase, trypsin.

10. A method according to claim 3 wherein the active moiety is a kinase and the interaction is with a kinase substrate or inhibitor.

11. A method according to claim 3 wherein the active moiety is a receptor and the interaction is with a ligand.

12. A method according to claim 3 wherein the active moiety is an antibody and the interaction is with an antigen or antigenic epitope.

13. A method according to claim 3 wherein the mixture of compounds in all of the wells of primary library L1 contain all variations of a first two variable groups and each well contains a unique pair of the two other variable groups; and wherein the mixture of compounds in all of the wells of secondary library L2 contain all variations of the two other variable groups and each well contains a unique pair of the first two variable groups.

14. A method according to claim 13 wherein the primary library contains all variations of variable groups B and E, and unique pairs of variable groups C and D; and the secondary library contains all variations of variable groups C and D, and unique pairs of variable groups B and E.

format according to claim 3 or 4, applying an active moiety to the wells, screening for an interaction of the active moiety with one or more compounds of a mixture in a well of primary library L1 and secondary library L2 and deducing from the respective L1 and L2 well positions a unique active compound formula.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10		
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10		
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10		
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10		
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10		
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10		
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10		
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10		

Figure 1

First Example of Library Matrix where n=1

Component Distribution in Plate 1, Library 1 (n=1)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-1} E_{1-1}	B_{1-2} E_{1-2}	B_{1-3} E_{1-3}	B_{1-4} E_{1-4}	B_{1-5} E_{1-5}	B_{1-6} E_{1-6}	B_{1-7} E_{1-7}	B_{1-8} E_{1-8}	B_{1-9} E_{1-9}	B_{1-10} E_{1-10}
D2	B_{1-1} E_{1-1}	B_{1-2} E_{1-2}	B_{1-3} E_{1-3}	B_{1-4} E_{1-4}	B_{1-5} E_{1-5}	B_{1-6} E_{1-6}	B_{1-7} E_{1-7}	B_{1-8} E_{1-8}	B_{1-9} E_{1-9}	B_{1-10} E_{1-10}
D3	B_{1-1} E_{1-1}	B_{1-2} E_{1-2}	B_{1-3} E_{1-3}	B_{1-4} E_{1-4}	B_{1-5} E_{1-5}	B_{1-6} E_{1-6}	B_{1-7} E_{1-7}	B_{1-8} E_{1-8}	B_{1-9} E_{1-9}	B_{1-10} E_{1-10}
D4	B_{1-1} E_{1-1}	B_{1-2} E_{1-2}	B_{1-3} E_{1-3}	B_{1-4} E_{1-4}	B_{1-5} E_{1-5}	B_{1-6} E_{1-6}	B_{1-7} E_{1-7}	B_{1-8} E_{1-8}	B_{1-9} E_{1-9}	B_{1-10} E_{1-10}
D5	B_{1-1} E_{1-1}	B_{1-2} E_{1-2}	B_{1-3} E_{1-3}	B_{1-4} E_{1-4}	B_{1-5} E_{1-5}	B_{1-6} E_{1-6}	B_{1-7} E_{1-7}	B_{1-8} E_{1-8}	B_{1-9} E_{1-9}	B_{1-10} E_{1-10}
D6	B_{1-1} E_{1-1}	B_{1-2} E_{1-2}	B_{1-3} E_{1-3}	B_{1-4} E_{1-4}	B_{1-5} E_{1-5}	B_{1-6} E_{1-6}	B_{1-7} E_{1-7}	B_{1-8} E_{1-8}	B_{1-9} E_{1-9}	B_{1-10} E_{1-10}
D7	B_{1-1} E_{1-1}	B_{1-2} E_{1-2}	B_{1-3} E_{1-3}	B_{1-4} E_{1-4}	B_{1-5} E_{1-5}	B_{1-6} E_{1-6}	B_{1-7} E_{1-7}	B_{1-8} E_{1-8}	B_{1-9} E_{1-9}	B_{1-10} E_{1-10}
D8	B_{1-1} E_{1-1}	B_{1-2} E_{1-2}	B_{1-3} E_{1-3}	B_{1-4} E_{1-4}	B_{1-5} E_{1-5}	B_{1-6} E_{1-6}	B_{1-7} E_{1-7}	B_{1-8} E_{1-8}	B_{1-9} E_{1-9}	B_{1-10} E_{1-10}

Figure 2

Component Location in Plate 1, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	$C_{1,10}$ $D_{1,2}$	$C_{1,11}$ $D_{1,3}$	$C_{1,12}$ $D_{1,4}$	$C_{1,13}$ $D_{1,5}$	$C_{1,14}$ $D_{1,6}$	$C_{1,15}$ $D_{1,7}$	$C_{1,16}$ $D_{1,8}$	$C_{1,17}$ $D_{1,9}$	$C_{1,18}$ $D_{1,10}$	$C_{1,19}$ $D_{1,11}$
E2	$C_{1,20}$ $D_{1,2}$	$C_{1,21}$ $D_{1,3}$	$C_{1,22}$ $D_{1,4}$	$C_{1,23}$ $D_{1,5}$	$C_{1,24}$ $D_{1,6}$	$C_{1,25}$ $D_{1,7}$	$C_{1,26}$ $D_{1,8}$	$C_{1,27}$ $D_{1,9}$	$C_{1,28}$ $D_{1,10}$	$C_{1,29}$ $D_{1,11}$

Figure 3

Component Location in Plate 2, Library 2 (n=1..

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E2	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}

Figure 4

Component Location in Plate 3, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E2	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}

Figure 5

Component Location in Plate 4, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{n-8}	C_{1-10} D_{n-9}	C_{1-10} D_{n-10}	C_{1-10} D_{n-11}	C_{1-10} D_{n-12}	C_{1-10} D_{n-1}	C_{1-10} D_{n-2}	C_{1-10} D_{n-3}	C_{1-10} D_{n-4}	C_{1-10} D_{n-5}
E2	C_{1-10} D_{n-6}	C_{1-10} D_{n-7}	C_{1-10} D_{n-8}	C_{1-10} D_{n-9}	C_{1-10} D_{n-10}	C_{1-10} D_{n-11}	C_{1-10} D_{n-12}	C_{1-10} D_{n-1}	C_{1-10} D_{n-2}	C_{1-10} D_{n-3}

Figure 6

Example Library where n=4

Component Distribution in Plate 1, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}
D2	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}
D3	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}
D4	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}
D5	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}
D6	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}
D7	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}
D8	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}	B_{1-1C} E_{1-2}

Component Location in Plate 2, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D2	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D3	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D4	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D5	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D6	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D7	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D8	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}

Figure 8

Component Location in Plate 3, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D2	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D3	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D4	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D5	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D6	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D7	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D8	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}

Figure 9

Component Location in Plate 4, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-10} E_{n-8}	B_{1-10} E_{n-9}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}
D2	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}
D3	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}
D4	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}
D5	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}
D6	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}
D7	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}
D8	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}	B_{1-10} E_{n-8}

Figure 10

Component Location in Plate 1, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}
	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}
E2	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}
	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}
E3	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}
	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}
E4	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}
	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}
E5	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}
	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}
E6	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}
	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}
E7	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}
	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}
E8	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}	C_{1-10}
	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}	D_{1-2}

Component Location in Plate 1, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}
E2	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}
E3	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}
E4	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}
E5	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}
E6	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}
E7	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}
E8	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}	C_{1+10} D_{3+4}

Component Location in Plate 3, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E2	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E3	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E4	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E5	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E6	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E7	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E8	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}

Component Location in Plate 4, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E2	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E3	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E4	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E5	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E6	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E7	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E8	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}

Figure 14.

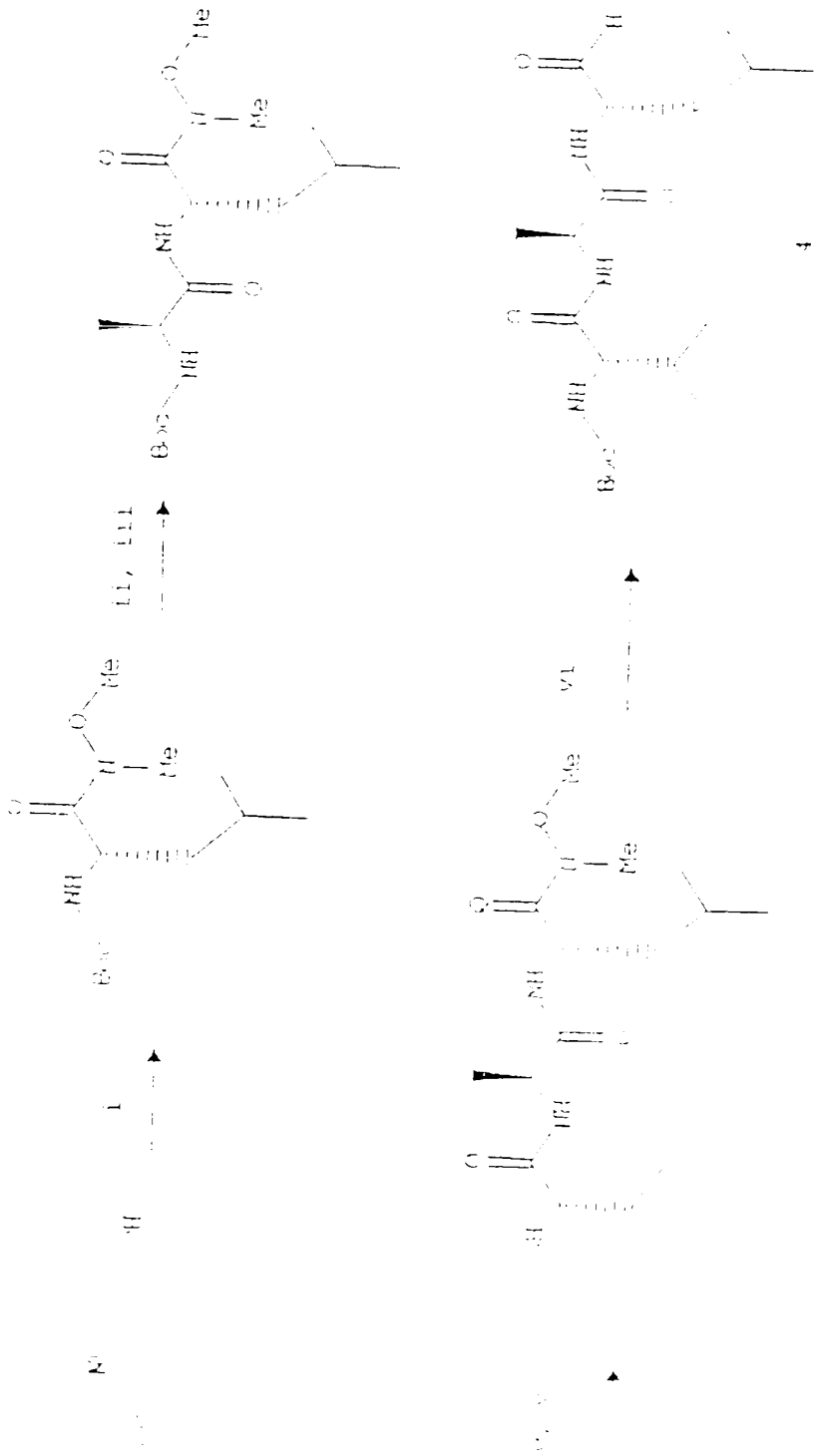
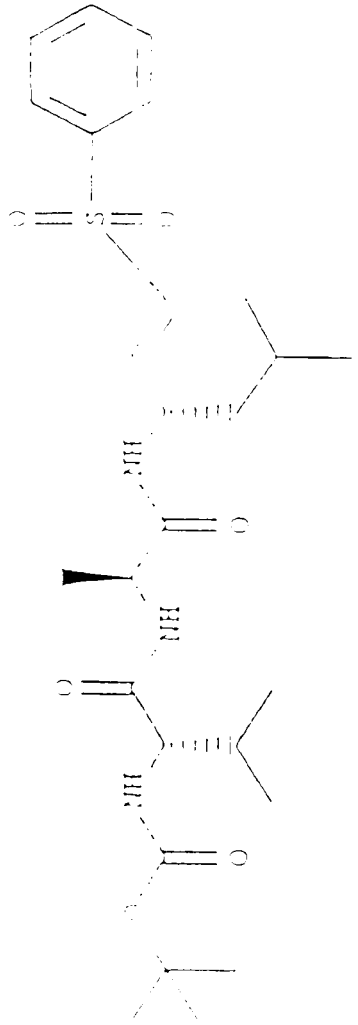
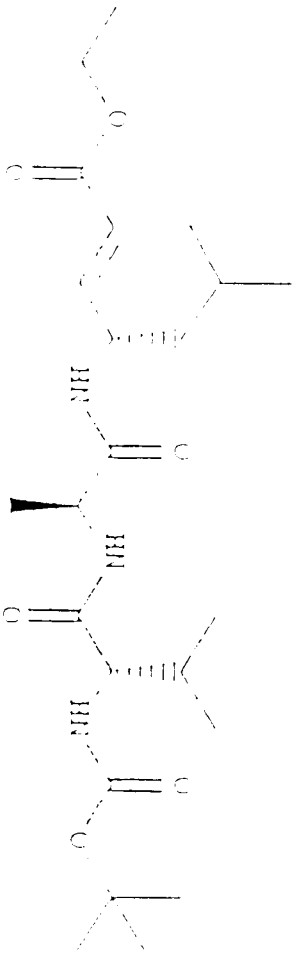


Figure 15



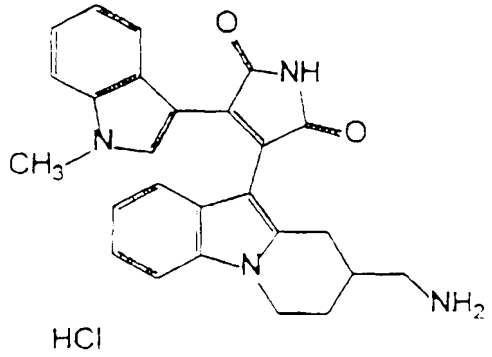
5'



6'

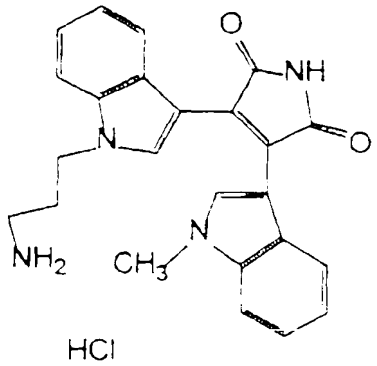
Figure 16

A



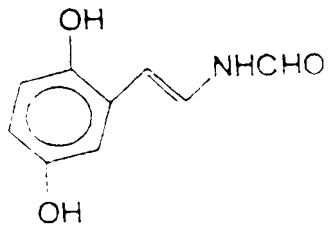
m.w. 460.965

B



m.w. 434.927

Figure 17



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/01158

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K1/04 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	M. MELDAL ET AL.: "Portion-mixing peptide libraries of quenched fluorogenic substrates for complete subsite mapping of endoprotease specificity" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 8, 12 April 1994, WASHINGTON US, pages 3314-3318, XP002042095 see page 3317, right-hand column, paragraph 2 - page 3318, right-hand column, paragraph 1 ---	1,2
A	WO 95 34575 A (SYNTHETIC PEPTIDES INC) 21 December 1995 see the whole document ---	1,3,9-12
	-/--	

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

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INTERNATIONAL SEARCH REPORT

Inte ...ional Application No
PCT/GB 97/01158

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	WO 94 05394 A (ARRIS PHARMACEUTICAL CORP) 17 March 1994 see claims; examples	1,3,9-12
A	B. Déprez et al.:" Self-decifering, orthogonal combinatorial libraries of soluble organic compounds: Discovery of a potent V2 vasopressin antagonist" in: XP002042096 Peptides 1994 Proceedings of the Twenty-Third European Symposium September 4-10, 1994, Braga, Portugal ed. HLS Maia; pub. ESCOM, Leiden, NL, 1995, pages 455-456	1,3,11
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		WO 9419694 A	01-09-94